

Inhibition of Cathepsin S Reduces Allogeneic T Cell Priming but Not Graft-versus-Host Disease Against Minor Histocompatibility Antigens

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Cathepsin (Cathepsin) S, L, and B proteases mediate antigen presentation on major histocompatibility complex (MHC) class II by degrading the invariant chain Ii, which blocks peptide loading. The ability of the Cathepsin S inhibitor LHVS (morpholinurea-leucine-homophenylalanine-vinylsulfone phenyl) to impede antigen presentation has led its development as a therapy for autoimmune diseases. There is substantial evidence that donor T cell recognition of host minor histocompatibility antigens (miHA) and subsequent destruction of host tissue mediates graft-versus-host disease (GVHD). We hypothesized that enzymes involved in antigen presentation may play a role in the development of GVHD. Using the C57BL/6 → BALB.B minor mismatch acute GVHD (aGVHD) model, we found that the cathepsin S activity of spleens from allogeneically transplanted mice were significantly increased 1 week after transplantation compared with syngeneic mice. Although LHVS decreased T cell priming responses against both single OVA antigen and miHA in vitro, LHVS did not reduce the severity of aGVHD. In fact, LHVS exacerbated a CD4⁺-T cell-dependent model of GVHD similar to chronic GVHD. This suggests that cytokines rather than T cells may mediate much of the damage in the aGVHD model and that therapeutics based on inhibition of antigen presentation for GVHD must be approached with caution.

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INTRODUCTION

Graft-versus-host disease (GVHD), secondary to T cell recognition of host minor histocompatibility antigens (miHA), is a major complication of blood and marrow transplantation (BMT). Although the standard treatment for this disease is immunosuppression,

the tendency of immunosuppressive agents to target T cells can lead to severe infections. Another approach is to utilize drugs with selective effects on antigen-presenting cells (APCs), thereby decreasing their ability to present antigen to and activate host-reactive T cells. In this regard, the 4-aminoquinolines, chloroquine, and hydroxychloroquine, block major histocompatibility complex (MHC) class II antigen processing and presentation in vitro. We have previously shown that chloroquine treatment of mice leads to decreased T cell responses and lowered incidence of GVHD [1,2]. Chloroquine derivatives are nonspecific; they raise endosomal pH, disrupting multiple endosomal enzymes and protein interactions [3,4] including antigen-processing proteases and Toll-like receptor (TLR)-ligand interactions. Chloroquine-mediated inhibition of GVHD may therefore work by downregulation of MHC II-mediated antigen presentation or by inhibiting TLR9 signaling, as the latter has been implicated in the pathology of GVHD [5]. However, phase III clinical trials have shown that chloroquine is not better than cyclosporin plus steroid at treating established GVHD [6,7]. A specific inhibitor of antigen presentation, given prophylactically, may be a more effective alternative to chloroquine.

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After the invariant chain (Ii) binds to MHC class II molecules in the endoplasmic reticulum, the complex is transferred to endosomes where the acidic environment activates proteases such as cathepsin L, B, and S that cleave Ii. The truncated form of Ii is still able to reduce the association of other peptides with the MHC II complex until after Cathepsin S converts it to class II-associated invariant chain peptide (CLIP). With the help of the MHC II-like molecule DM, CLIP can then be displaced by degraded antigen, after which the MHC II/antigen complex is transported to the cell surface. By controlling the pace of Ii degradation, Cathepsin S is thus able to influence the rate of MHC class II-mediated antigen presentation [8-10].

In mice, cathepsin S is active in professional antigen-presenting cells such as dendritic cells, B cells, and macrophages [8], whereas cathepsin L is active in thymic epithelial cells [11]. In humans and mice, cathepsin S also processes antigen in nonprofessional APCs [11,12]. Cathepsin S has been shown to be involved in the presentation of endogenous antigens such as IgM, β_2 -M, or E α 52-68 [8,13].

LHVS (morpholinurea-leucine-homopenylalanine-vinylsulfone phenyl) inhibits cathepsin S activity [14,15] by direct interaction [16] with therapeutic effects on autoimmune diseases [8,17], whereas other cathepsin S inhibitors show potential as a therapeutic strategy for a mouse model of Sjögren syndrome [18]. In mixed lymphocyte responses, proliferation of I-A^d splenocytes in response to stimulation with splenocytes of cathepsin S^{-/-} I-A^b mice was reduced by 50% compared with cathepsin S^{+/+} mice [10]. However, the extent of Cathepsin S involvement on antigen processing is likely to be both haplotype- and peptide-dependent [9]. T cells are unaffected by LHVS treatment, as spleen cells from mice treated with LHVS exhibit a normal response to heterologous spleen cells [17]. Although LHVS inhibition of cathepsin S in autoimmune disease models shows decreased self-antigen-specific T cell proliferation, little is known about its effect on presentation of alloantigens and impact on tissue rejection as seen in acute or chronic forms of GVHD. We hypothesized that suppression of the MHC class II processing pathways by cathepsin inhibition would reduce GVHD. To investigate this, we used a lethal GVHD mouse model based on minor histocompatibility mismatches. We found significant elevation of cathepsin S activity at day 7 and cathepsin L/B activity at day 14 in allogeneic mice compared with syngeneic control after transplantation, suggesting that cathepsin S, L, and B may be required for miHA antigen processing in this model. Although LHVS-mediated Cathepsin S inhibition decreased both presentation of exogenous OVA antigen and presentation of endogenous MiHA, it did not inhibit lethal GVHD in an acute model. Interestingly, LHVS treatment exacer-

bated a CD4⁺-dependent model of chronic GVHD (cGVHD)-like disease.

MATERIALS AND METHODS

Mice

Five- to 6-week-old C57BL/6 (B6; H-2^b) and C.B10-H2^b/LiMcJ (BALB.B (BB); H-2^b) mice were obtained from The Charles River (Wilmington, MA) and Jackson Laboratories (Bar Harbor, ME). Mice were maintained as a breeding colony at the Child and Family Research Institute, Vancouver, Canada, housed under specific pathogen-free conditions in microisolators. All experiments were performed in accordance with the Canadian Council on Animal Care guidelines and with institute review board approval. OT-II TCR transgenic mice [19] were maintained by Dr. Dutz (UBC, Vancouver, Canada).

Reagents

LHVS, an irreversible, specific cathepsin S inhibitor was a gift from Arris Pharmaceuticals (South San Francisco, CA), and stock solutions were made in vehicle. LHVS was tested negative for endotoxin contamination with Limulus Amebocyte Lysate PYROGENT® Plus (Cambrex Bio Science, Walkersville, MD).

Measurement of Endogenous Cathepsin Activity

Spleen and lymph nodes from C57BL/6 mice were used to determine cathepsin S, B, and L activity, as previously described [18]. Briefly, tissues were gently homogenized in 0.25 M cold sucrose and spun down at $1,080 \times g$ for 20 minutes at 4°C. The collected supernatant was centrifuged at $25,000 \times g$ for 30 minutes at 4°C, and the resulting pellet was suspended in 50 mM acetate buffer (pH 5.0) for cathepsin B and L or in 0.1 M potassium phosphate buffer (containing 5 mM EDTA-2Na, 5 mM DTT and 0.01% Triton X-100, pH = 6.5) for cathepsin S. After the suspension fluid was freeze-thawed three times to disrupt lysosomal membranes, the fluid was centrifuged at $25,000 \times g$ for 20 minutes at 4°C, and the supernatant was used as the lysosome fraction. The isolated lysosome fraction was preincubated with specific inhibitors (CA074 Peptide International, California; Z-FY(t-Bu)DMK; Calbiochem, San Diego, CA) for 5 minutes for cathepsin B and cathepsin L, 45 minutes for cathepsin S at 37°C, and the fluorescence was then measured with Z-Phe-Arg-methyl coumarylamide as a substrate (final 10 μ M; Peptide International, Louisville, KY) for cathepsin B and L and with Z-Val-Val-Arg-methyl coumarylamide (final 5 μ M) for cathepsin S. The fluorescence of the liberated 7-amino-4-methylcoumarin was measured by fluorescence spectrophotometer,

with emission at 460 nm and excitation at 355 nm. One unit of enzyme was defined as the amount, which degraded 1 μ mol of substrate per minute.

In Vivo Treatment with a Cathepsin S-Specific Inhibitor

Mice were given either LHVS or vehicle by i.p. injection at different concentrations (50 or 100 mg/kg) for 2 weeks (three times per week). Spleen and lymph nodes were used for cathepsin L, cathepsin B, and cathepsin S assays. Mice undergoing BMT in the GVHD models were given inhibitor or control three times weekly i.p. starting 2 weeks before BMT until the end of the experiment.

Proliferation Assay

For CD3 monoclonal antibody (CD3mAb) stimulation, 96-well plates were coated with anti-CD3mAb (10 μ g/mL) and incubated at 37°C for 90 minutes before washing. C57BL/6 splenocytes (2×10^5 /well) were cultured in the coated plates with LHVS or vehicle for 24 hours before pulsing with 1.0 μ Ci [3 H]thymidine (Amersham, Oakville, Ontario, Canada) for a further 18 hours.

For measurement of ovalbumin-mediated stimulation, CD4 $^+$ T cells (5×10^4 /well) were purified from OT-II splenocytes by negative selection as follows: CD8, CD11b (Mac-1), CD45R/B220, Ly-6G/C (Gr-1), and TER119-expressing cells were removed by the CD4 $^+$ T Cell Enrichment Kit StemSep® Mouse from StemCell (Vancouver, Canada). These CD4 $^+$ T cells were combined with dendritic cells (DC) isolated by positive selection of CD11 $^+$ c (EasySep®, StemCell Technologies Inc.) from splenocytes (5×10^4 /well) of C57BL/6 mice treated with 50 or 100 mg/kg of LHVS or the equivalent volume of vehicle three times per week for 2 weeks. The mixed cultures were incubated with ovalbumin protein (100 μ g/mL) or OVA₃₂₃₋₃₃₉ peptide (1 μ g/mL) for 72 hours and then pulsed with [3 H]thymidine for another 18 hours. DNA was then harvested using a Mach III M harvester (Tomtec, Hamden, CT), and [3 H] thymidine incorporation was counted on a 1450 Microbeta liquid scintillation counter (Wallac Trilux, Turku, Finland).

Cytokine Production

Plasma was collected from syngeneic and allogeneic mice at day 7 and day 14 after transplantation and stored at -80°C until analysis. Interferon (IFN)- γ , tumor necrosis factor (TNF)- α , and interleukin (IL)-6 levels were determined using the mouse inflammation cytometric bead array (CBA) kit (BD Pharmingen, San Diego, CA) following the manufacturer's protocol.

OT-II LN cells (1×10^6 /well) and irradiated C57BL/6 splenocytes (1×10^6 /well) from mice treated with LHVS (100 mg/kg) or vehicle were cultured with

OVA protein (100 μ g/mL) or OVA₃₂₃₋₃₃₉ peptide (1 μ g/mL) for 72 hours at 37°C in a 24-well plate in 10% RPMI media. The supernatant was collected 24 hours after incubation; IL-2 and IFN- γ levels were measured by ELISA (BD Pharmingen). Samples and standards were run in duplicate.

T Cell Priming for Minor Histocompatibility Antigens and Proliferation Assay

C57BL/6 mice were administered LHVS (100 mg/kg) or vehicle three times weekly for 2 weeks, followed by two intraperitoneal injections of 2.5×10^7 BALB.B splenocytes.

After injection of LHVS or vehicle for another 4 to 8 weeks (three times weekly), responder C57BL/6 splenocytes (4×10^5 cells/well) were cultured in a 96-well plate in 10% RPMI media with mitomycin C-treated stimulator BALB.B splenocytes at an R:S ratio of 1:1, 2:1, 4:1 for 96 hours and then pulsed with 1.0 μ Ci [3 H]thymidine for another 18 hours. Data are presented as the mean difference, with standard error of the mean (SEM) bars in stimulated and unstimulated cultures (Δ CPM).

BMT

Acute GVHD (aGVHD) model: recipient BALB.B female mice between 8 to 12 weeks were irradiated at 680 cGy (300 kV; 2 Gy/min) before injection with 8×10^6 BM and 2×10^7 spleen cells from C57BL/6 female mice within 24 hours. In the second GVHD model, which resembles cGVHD, recipient BALB.B female mice were injected with either 5×10^6 T cell-depleted bone marrow (TCD-BM) or TCD-BM cells plus $1-2 \times 10^6$ naive CD4 (CD62L $^{\text{high}}$ CD44 $^{-/\text{low}}$ CD25 $^{-}$) T cells. Naive T cells were isolated by flow-cytometric sorting as described in the information to follow. T cells were depleted from bone marrow using biotinylated anti-Thy1.2 antibody (EasySep, StemCell Technologies Inc.). Evaluation of GVHD was based on well established criteria consisting of several categories: overall ill appearance (hunching, ruffled fur), wasting, fur loss, and wrinkling of the skin. Disease was quantified in the acute model by scoring from 0 to 2 in all categories as previously described [20]. To quantitate disease in the cGVHD-like model, mice were scored blindly as follows: 0, healthy; 1, skin lesions with alopecia less than 1 cm 2 ; 2, skin lesions with alopecia 1 to 2 cm 2 ; 3, skin lesions with alopecia more than 2 cm 2 . Additionally, the mice were assigned 0.3 points each for skin disease (lesion or scaling) on the ears, tail, and paws (0-3.9 points) three times per week according to established protocols [20,21]. Mice were sacrificed if their weight dropped below 20% of their starting weight according to criteria established by the University of British Columbia Ethical Review Committee.

Sorting of Naive CD4⁺ T Cells

Cells pooled from spleen and lymph nodes were first stained with Cy-Chrome-conjugated antimouse CD4 (RM4-5), fluorescein isothiocyanate (FITC)-conjugated anti-CD25 (7D4), APC-conjugated anti-CD44 (IM7), and phycoerythrin (PE)-conjugated anti-CD62L (MEL-14). All antibodies were purchased from BD Pharmingen (San Jose, CA). The stained cells were sorted into naive (CD62L^{high}CD44^{low}) CD25⁻CD4⁺ T cells using a FACS Aria equipped with FACSDiva software (Becton Dickinson, San Jose, CA). The purity of positively selected naive CD4⁺ T cells was >97%.

Flow Cytometric Analysis

Four-color flow cytometry was performed on a FACSCalibur™ flow cytometer (Becton Dickinson). For analysis of cell surface molecules, samples were labeled with directly conjugated fluorescent antibodies at 4°C for 30 minutes. The samples were then washed and suspended in 1.5% paraformaldehyde and analyzed within 24 hours. All antibodies were obtained from BD Pharmingen (San Diego, CA). Relevantly labeled isotype control antibodies were included in all experiments. Analysis was performed using FLOWJO software (Tree Star, San Carlos, CA).

Engraftment

Marker D8Mit224 was used for detection of donor cells in the C57BL/6 → BALB.B transplantations. Polymerase chain reaction (PCR) was run with 250 ng of genomic DNA in 25-μL reaction volumes for 40 cycles.

Quantitative PCR

Expression levels of cathepsin S and internal reference 18sRNA was measured by quantitative PCR (qPCR). Total RNA was extracted from sorted macrophages (CD11b⁺CD11c⁻), dendritic cells (CD11b⁺CD11c⁺), and B cells (B220⁺CD11c⁻) using Trizol reagent (Gibco BRL, Rockville, MD). Because of the scarcity of dendritic cells, myeloid and lymphoid DC were analyzed together as total dendritic cells. cDNA was synthesized with equal amounts of total RNA using oligo dT primers and Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). Real-time qPCR was performed using the Lumix system (Invitrogen Life Technologies). Amplification and product detection was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) as recommended by the manufacturer. The quantity of cDNA for each RNA sample was normalized to the quantity of 18sRNA cDNA in each sample, and relative expression was determined using the $\Delta\Delta C_t$ (threshold cycle) method according to the manufacturer's protocol. LUX Fluor-

ogenic Primer (Invitrogen Corp.) specific for each mRNA was designed using Invitrogen's D-LUX Designer Software. Cathepsin S was assayed in triplicate using the following primers (FAM-labeled Lux primer; Invitrogen) F: 5'-GCAGACGCTTCCTATCCCTACAA-3', R: 5'-GGAGCTGAATGTACCTTGAACA-3'. The FAM-labeled mouse 18sRNA Certified LUX Primer Set (Invitrogen) was used to amplify the controls.

Statistical Analysis

Survival curves were plotted using Kaplan-Meier estimates and analyzed by log-rank test. For statistical analysis, the Mann-Whitney *U*-test was used, except for the results presented in Figures 1C and 2 that were analyzed using the paired *t*-test. All results are presented as mean ± standard error (SE). Descriptive statistics were generated on all data using Prism version 4 for PC (GraphPad Software, San Diego, CA).

RESULTS

Cathepsin S Activity in an miHA Mismatched GVHD Murine Model

To assess the role of cathepsin S in the development of GVHD, we first evaluated cathepsin activities in the spleen and lymph nodes from mice transplanted with miHA disparate (B6 → BB) and syngeneic (BB → BB; negative control) donor cells. As shown in Figure 1A, splenic cathepsin S activity in mice receiving MiHA disparate (C57BL/6) donor cells was significantly higher than mice receiving syngeneic (BALB.B) donor cells 7 days after transplantation (mean ± SEM, 174.0 ± 16.9 versus 49.7 ± 17.0 mU/mg protein, *P* < .01). At 14 days posttransplantation, there is no significant difference in cathepsin S activity. In contrast, cathepsins B and L were not different at the 7-day time point but showed increased activity in the spleen 14 days after transplantation (Figure 1B and C). (Syngeneic versus MiHA disparate activities: cathepsin B is 656.5 ± 61.4 versus 2292.0 ± 103.9 mU/mg protein; *P* < .01; cathepsin L is 9.1 ± 3.7 versus 33.5 ± 3.5 mU/mg protein; *P* < .01).

Because we used total splenic cells as a source of cathepsins, and cathepsin S is expressed in several types of APCs such as B cells, DCs, and macrophages, we sought to determine if one particular type of APC was the source of increased cathepsin S activity at day 7 after allogeneic transplantation. Determination of absolute B cell, DC, and macrophage populations in the spleen at day 7 showed that although all populations increased after allogeneic compared with syngeneic transplantations, macrophages were the most numerous APC population in allotransplanted mice at day 7 (Figure 1D). qPCR analyses showed that relative cathepsin S mRNA levels were 1.9, 4.5, and 5.8 times higher in macrophages, B cells, and DC

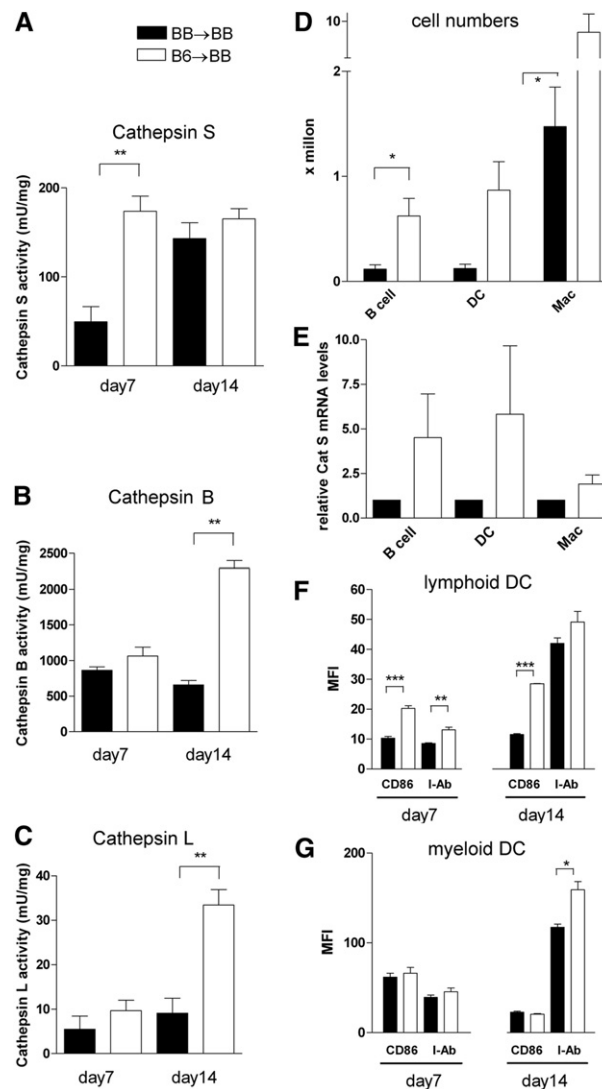


Figure 1. Endogenous splenic Cathepsin S activity after transplantation. (A-C) Splenic Cathepsin S, B, and L activities were determined 7 and 14 days posttransplantation. (D) Total numbers of B cells ($B220^+CD11c^-$), DCs ($CD11c^+$), and macrophages (Mac, $CD11b^+CD11c^-$) in the spleen were determined at day 7 posttransplantation. (E) Also at day 7 posttransplantation, FACS-sorted splenic B cells, macrophages ($CD11b^+CD11c^-$), and DCs were analyzed for relative cathepsin S mRNA levels by quantitative PCR. Data are expressed as a ratio of mRNA relative to 18sRNA. (F and G) Surface marker analysis of lymphoid ($CD11b^-CD11c^+$) and myeloid ($CD11b^+CD11c^+$) splenic dendritic cells from transplanted mice. MFI, mean fluorescent intensity. All results are representative of three independent experiments. Black bar (BB → BB) indicates mice receiving syngeneic donor cells, whereas white bar (B6 → BB) indicates mice receiving allogeneic donor cells. The data show mean \pm SEM. *, **, *** indicates $P < .05$, .01, .001, respectively.

($CD11c^+/CD11b^+$), respectively, in allotransplanted mice compared with mice receiving syngeneic donor cells (Figure 1E), suggesting that all APCs were contributing the increased cathepsin S activity at day 7. Flow cytometric analyses showed that CD86 and/or MHC class II (I-A^b) expression on DCs were upregulated in mice receiving allogeneic donor cells compared with control. Lymphoid DCs showed an increase in both CD86 and MHC II expression (Figure 1F), whereas only MCH II expression increased in myeloid DCs after 14 days (Figure 1G).

As inflammatory cytokines such as TNF- α , IL-6, and IFN- γ have been reported to influence cathepsin activity in macrophages and DCs [22-24], we evaluated the association between plasma cytokine

profiles and cathepsin expression in our GVHD model. Plasma levels of TNF- α , IL-6, and IFN- γ were determined on days 7 and 14 after syngeneic and allogeneic transplantation (Table 1 and Figure 2A-C). With the exception of IL-6 at day 14, all plasma derived from allogeneically transplanted mice had significantly higher levels of cytokines than the syngeneic controls. Although both INF- γ and IL-6 were higher 7 days posttransplantation than 14 days posttransplantation, TNF- α levels were increased between day 7 and day 14. Although IL-6 and IFN- γ may have contributed to the initial increase in cathepsin S activity, it remains high at 14 days posttransplantation, whereas levels of these cytokines decrease. TNF- α levels alone increase from day 7 to day 14

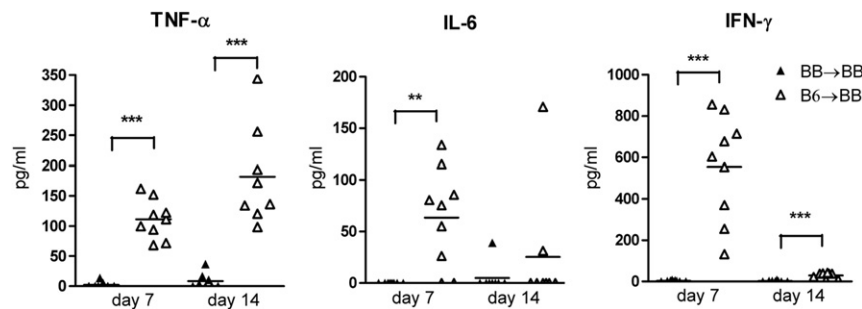


Figure 2. Plasma cytokines 7 days posttransplantation. Plasma cytokines TNF- α , IL-6, and IFN- γ present in syngeneic and allogeneic mice 7 days ($n = 9$) and 14 days ($n = 8$) after transplantation. All results are presented as mean \pm SEM for a minimum of three mice/group. *** indicates $P < .001$, ** $P < .01$.

posttransplantation, similar to the activity of cathepsins B and L.

Cathepsin Activities In Vivo

We have previously shown that therapeutic interventions that induce decreased in vivo T cell priming responses to antigens including MiHC correlate with the ability to inhibit GVHD in murine models [1]. Having shown that cathepsin S is increased in macrophages, DCs, and B cells day 7 after transplantation, we evaluated whether the cathepsin inhibitor LHVS could affect cathepsin activity in vivo, inhibit T cell antigen-specific priming to exogenous antigens, and reduce GVHD. LHVS affects cathepsin activity in vitro, in a dose-dependent manner, with inhibition of cathepsin S at lower concentrations (100 nM) and cathepsin B and L activity at higher concentrations ($>1 \mu\text{M}$) (data not shown). LHVS was administered by i.p. injection three times weekly for 2 weeks followed by measurement of cathepsin S, B, and L activity in spleen- and lymph node-derived lymphocytes. As shown in Figure 3, splenic and lymph node Cathepsin S activity was significantly decreased in mice treated with 50 mg and 100 mg/kg LHVS. (Splenocytes from vehicle-, LHVS50- and LHVS100-treated mice: 208.1 ± 15.8 , 63.7 ± 14.7 , 50.9 ± 18.1 mUnits/mg, respectively; lymph nodes from vehicle-, LHVS50-, and LHVS100-treated mice: 63.2 ± 25.4 , 14.5 ± 7.1 , 15.6 ± 7.1 mUnit/mg, respectively). Higher concentrations of LHVS (100 mg/kg) inhibited splenic cathepsin B activity (vehicle, LHVS50, and LHVS100:

991.6 ± 183.4 , 626.6 ± 97.8 , 313.8 ± 56.3 mUnit/mg, respectively) but not cathepsin L.

Effect of LHVS on In Vivo T Cell Priming to Minor Histocompatibility Antigens in GVHD Models (C57BL/6 anti-BALB.B)

Studies have shown that LHVS can inhibit exogenous antigen presentation in vitro and in vivo [9,17]. In preparation for in vivo T cell priming experiments, we first confirmed the ability of in vivo-administered LHVS to affect the ability of DCs to present exogenous antigens. Purified DC (CD11c $^{+}$) from mice treated three times weekly with LHVS or vehicle were removed after 2 weeks of treatment and evaluated for CD4 $^{+}$ OT-II transgenic T cell responses after pulsing with exogenous OVA protein. As shown in Figure 4A, OT-II CD4 $^{+}$ T cell proliferation was significantly inhibited after pulsing with DC derived from LHVS-treated mice compared with DC derived from vehicle-treated mice in a dose-dependent manner (vehicle, LHVS50, and LHVS100: 9363 ± 520 , 3794 ± 483 , 816 ± 136 ΔCPM). The lower dose of LHVS, which inhibits cathepsin S only, inhibited the T cell response to exogenous OVA protein by about 50%, whereas the higher dose of LHVS almost completely abolished OVA-dependent T cell responses.

Because multiple endogenous miHA are recognized in GVHD, we next evaluated the effect of LHVS on a T cell priming response against miHA using the previously established C57BL/6 anti-BALB.B miHA incompatible model (H-2 b identical, MiHA disparate) [1]. This response is dependent on both CD8 $^{+}$ and CD4 $^{+}$

Table 1. Cytokine Levels in Syngeneic versus Allogeneic Transplanted Mice

	Day 7			Day 14			Day 7 versus day 14
	Syngeneic	Allogeneic	P value	Syngeneic	Allogeneic	P value	P value
TNF- α	2.1 ± 1.5	110.9 ± 10.7	^a	8.4 ± 4.6	181.8 ± 29.1	^a	.03
IL-6	nd	63.6 ± 15.8	^b	4.9 ± 4.9	25.3 ± 21.1	ns	ns
IFN- γ	2.5 ± 0.9	555.1 ± 84.3	^a	2.0 ± 0.9	29.3 ± 5.1	^a	<.0001

TNF- α , IL-6, and IFN- γ were measured in plasma 7 ($n = 9$) and 14 days ($n = 8$) after syngeneic (BB \rightarrow BB) or allogeneic (B6 \rightarrow BB) transplantation. All results are presented as mean \pm SEM for a minimum of three mice/group.

^a $P < .001$.

^b $P < .01$.

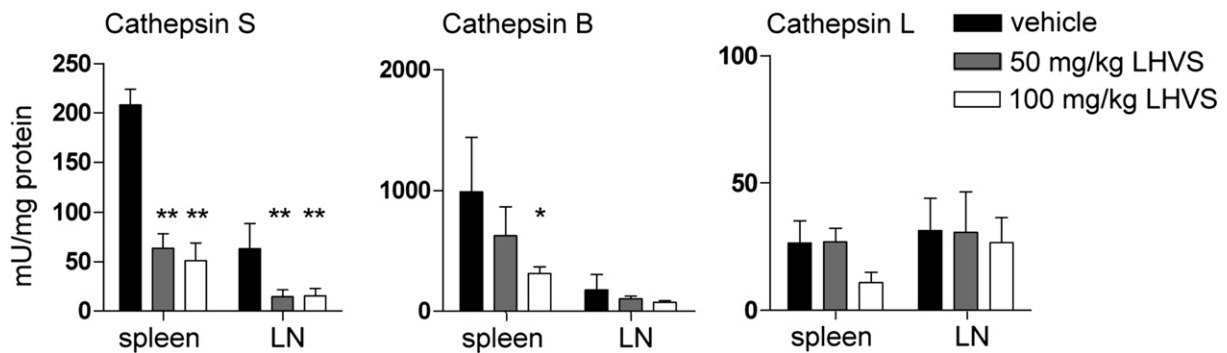


Figure 3. In vivo inhibition of splenic and lymph node cathepsin activity with LHVS. Cathepsin S, B, and L activities of spleen (SP) and lymph node (LN) were measured by quantitation of fluorescent cleavage product as described in the Methods section. Mice were given either 50 mg/kg (gray bar) or 100 mg/kg (white bar) of LHVS or vehicle (black bar) by i.p. injection for 2 weeks (three times per week). All results are presented as mean \pm SEM for a minimum of three mice/group. ** and * indicate $P < .01$ and $P < .05$, respectively.

T cells [1,25-27]. C57BL/6 mice were administered 100 mg/kg LHVS or vehicle three times weekly for 2 weeks before intraperitoneal injection of 2.5×10^7 BALB.B splenocytes. Administration of LHVS was continued three times per week for 4 to 8 weeks before evaluating splenic T cell responses. Splenocytes derived from C57BL/6 mice were then rechallenged in vitro with mitomycin C-treated BALB.B stimulators. Primed C57BL/6 anti-BALB.B T cell responses were significantly decreased in the LHVS-treated group compared with those in the vehicle-treated group (responder B6, stimulator BALB.B; vehicle 15107 ± 2790 Δ CPM, LHVS 6396 ± 1551 Δ CPM; $P < .01$), whereas treatment of splenocytes from both groups with ConA failed to induce similar differences in the responder cells (Figure 4B).

Effect of LHVS Treatment on the Development of GVHD in an Acute Murine miHA-Mismatched GVHD Model

Having established that LHVS treatment can significantly reduce T cell priming to miHA, we evalu-

ated the effect of LHVS treatment on aGVHD secondary to miHA disparity. Using the C57BL/6 \rightarrow BALB.B model, BALB.B recipient mice were treated three times per week with LHVS or an equivalent volume of vehicle starting 2 weeks before transplantation to ensure initial inhibition of MHC class II presentation. Treatment was continued at the identical dosing and frequency until the end of the experiment. All transplanted mice achieved complete chimerism in peripheral blood, confirmed by PCR 14 days after transplantation (data not shown). GVHD was measured by survival, weight loss, and aGVHD score (Figure 5A-C). Syngeneic transplanted mice BALB.B \rightarrow BALB.B all survived and had a transient weight loss associated with the myeloablation as well as a GVHD score as high as 2 at day 8 (because of the transient weight loss) before returning to 0. As expected, allogeneic transplanted mice treated with vehicle all developed GVHD with significant weight loss, 100% fatality by day 25, and a rising GVHD score. Of the LHVS-treated mice, all developed GVHD, and only one survived until the end of the experiment with severe

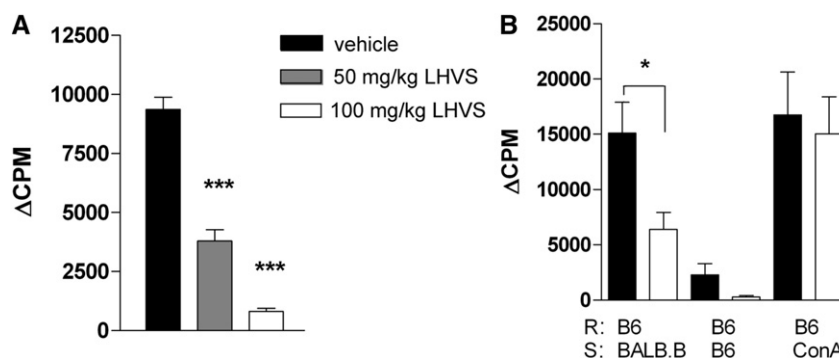


Figure 4. The effect of LHVS treatment in vivo on T cell proliferative responses to exogenous and minor histocompatibility antigens. (A) CD4⁺ T cells (5×10^4 /well) purified from OT-II splenocytes and DC isolated from C57BL/6 splenocytes (5×10^4 /well) treated with LHVS or vehicle (vehicle) three times weekly for 2 weeks were cultured with 100 μ g/mL ovalbumin protein for 72 hours and then pulsed with [³H]thymidine for another 18 hours before harvesting and determination of incorporation levels. (B) C57BL/6 mice were administered 100 mg/kg LHVS as described previously, followed by two intraperitoneal injections of BALB.B splenocytes (2 weeks apart). Administration of LHVS or vehicle was continued three times per week until sacrifice at 4 to 8 weeks posttransplantation. Responder C57BL/6 splenocytes were cultured with stimulating BALB.B splenocytes at an R:S ratio of 4:1 for 96 hours. T cell proliferation was measured by [³H] thymidine incorporation as described previously. Data are presented as the mean difference, with standard error of the mean (SEM) bars in stimulated and unstimulated cultures (Δ CPM). Data shown are the mean of three independent experiments, each group containing two to four mice. * and *** indicates $P < .05$ and $P < .001$, respectively.

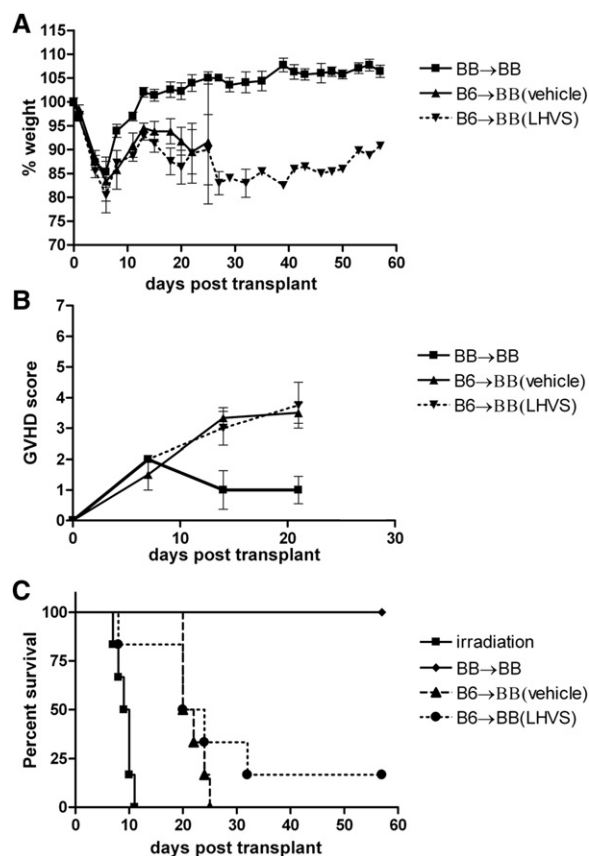


Figure 5. Effect of LHVS treatment on development of acute GVHD in a C57BL/6 → BALB.B miHA mismatch model. BALB.B recipients were administered 100 mg/kg LHVS or vehicle three times weekly starting 2 weeks before transplantation and continuing until the end of the experiment. After irradiation, recipients were injected with either 8×10^6 BM and 2×10^7 spleen cells from C57BL/6 mice or from BALB.B mice (syngeneic control). A group of mice not injected with cells after irradiation served as an irradiation control. Results are shown as mean \pm SEM from three different experiments; each group consisted of at least six mice at the time of transplantation. (A) Weight loss (note that at day 57, only one mouse was still alive in the allogeneic group treated with vehicle). (B) GVHD score. (C) Survival. Each experiment consisted of groups of at least six mice from two separate experiments.

cutaneous inflammation. No significant difference was seen in weight loss, survival, or GVHD scores in mice treated with vehicle versus those treated with LHVS. These results indicate that (1) LHVS is not more toxic than the vehicle (DMSO) after transplantation and (2) that LHVS-mediated cathepsin inhibition does not prevent the development of aGVHD.

Effect of LHVS Treatment on a CD4⁺ T Cell-Dependent Form of Chronic GVHD-Like Disease

The inability of LHVS to inhibit development of GVHD supports the relatively minor role of CD4⁺ T cell responses in the acute C57BL/6 → BALB.B model, which appears to be driven mainly by cytokine release and miHA-reactive donor CD8⁺ T cells [28-31]. As it has been shown that CD4⁺ T cells

mediate a disease similar to cGVHD in a B10.D2 → BALB/c mismatch model [32-34], we adapted this method to our GVHD model. T cell-depleted bone marrow was used to reduce aGVHD [29], and a cutaneous disease similar to chronic human GVHD was induced in BALB.B mice by transplantation of CD62L⁺CD44⁺ naive T cells from C57BL/6 mice. Splenic extracts of allogeneically transplanted mice treated with LHVS or vehicle were examined for cathepsin S activity to confirm in vivo inhibition in this model. As shown in Figure 6A, LHVS significantly suppressed cathepsin S activity in vivo after BMT ($51.7\% \pm 4.9\%$ of control; $P < .001$, *t* test). Figure 6B shows that mice injected with naive CD4⁺ T cells exhibited significant weight loss in comparison to mice injected with TCD-BM only ($P < .0001$, Mann-Whitney). However, cathepsin S inhibition with LHVS did not ameliorate symptoms of GVHD in this model. Serum IFN- γ and TNF- α levels in LHVS- versus vehicle-treated mice measured 14 days posttransplantation did not differ significantly: IFN- γ 10.9 ± 2.6 versus 8.2 ± 2.1 pg/mL; TNF- α 57.3 ± 10.4 versus 47.4 ± 14.7 , $n = 4$ versus 8, respectively. Mice treated with LHVS lost more weight ($P < .05$, Mann-Whitney) and, 4 weeks posttransplantation, exhibited more mortality ($P < .005$, log-rank test). Also, mice given LHVS instead of vehicle appeared to have worse skin pathology (although this difference was not significant) compared with mice given vehicle (Figure 6B and C). Determination of regulatory T cells (Treg) proportions (CD3⁺CD4⁺CD25⁺FoxP3⁺ cells versus CD3⁺CD4⁺ cells) from the peripheral blood mice 2 weeks posttransplantation revealed no significant differences between LHVS- and vehicle-treated mice, although the amount of mice surveyed was small ($n = 2-3$ mice per group; vehicle- versus LHVS-treated mice: 0.91 ± 0.44 versus 1.24 ± 0.28 ; % of CD3⁺ cells).

DISCUSSION

In this study, we found that cathepsin S, B, and L activities were elevated at different time points after transplantation in mice that develop GVHD (Figure 1). Elevated cathepsin S activity at day 7 may be the result of elevated IFN- γ , which can increase cathepsin activity [22-24]; however IFN- γ levels decrease by day 14, whereas cathepsin S remains elevated, indicating that other factors or cytokines are involved in the maintenance of high cathepsin S activity. IFN- γ is also known to decrease cathepsin L activity in macrophages [23], which is consistent with our observation that cathepsin L is higher after 14 days than after 7 days. Intriguingly, we found that splenic cathepsin L activity was significantly increased in allogeneic mice 14 days after transplantation (Figure 1A). It seems plausible that increased splenic

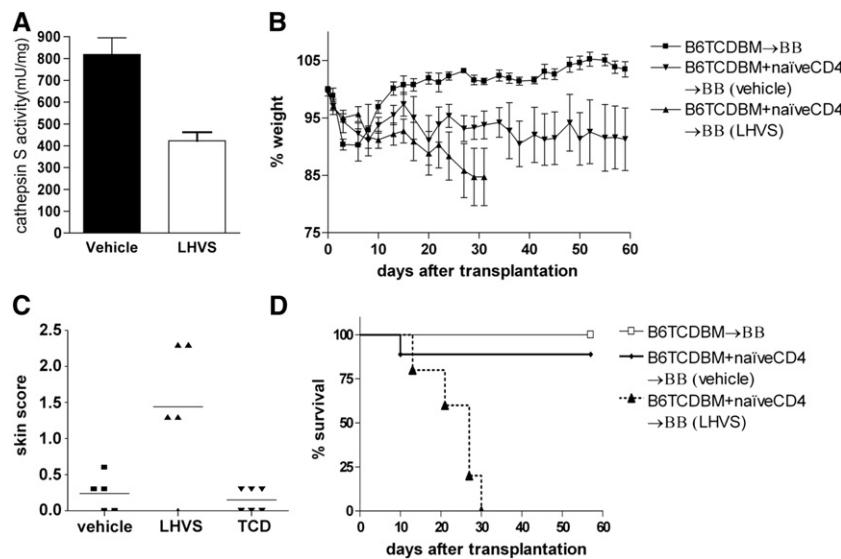


Figure 6. Effect of LHVS on a naive CD4⁺ T cell-mediated cGVHD model. Irradiated BALB.B recipients were injected with 5×10^6 C57BL/6 T cell-depleted BM cells (B6TCDBM) alone or in combination with naive CD4 (CD62L^{high} CD44^{low} CD25⁺ T cells). The recipients were administered either 100 mg/kg of LHVS or an equivalent volume of vehicle three times weekly starting at 2 weeks before transplantation and continuing until the end of the experiment. (A) Confirmation of cathepsin S inhibition in allogeneically transplanted mice. Mice were treated with 100 mg/kg LHVS or vehicle three times weekly beginning 14 days before BMT until the end of the experiment; splenic cathepsin activity was measured at 14 days post-BMT. (B) Mean weight loss \pm SEM; weight loss is expressed as a percentage of starting weight. (C) Skin pathology; vehicle, or LHVS-treated recipients were scored the end point (day 28). (D) Survival curve. These experiments were done twice and results were pooled; all groups consisted of at least five mice.

cathepsin B and L activity was induced by allogeneic antigen, which is supported by previous reports showing foreign antigens elevate active cathepsin L in the spleen and cathepsin B in DCs [35,36].

GVHD is the result of injury to host tissue caused by alloreactive donor T cells [37,38]. Memory T cells are much less efficient than naive T cells at causing GVHD [34,39-41], suggesting that priming by APCs in the host is crucial for generation of pathological T cell populations. GVHD following MHC-identical HSC transplantation is the result of miHC disparities following priming of donor-derived T cells by host or donor-derived APC-presenting host antigen. Priming of naive CD8⁺-T cells, a harbinger of immune-mediated destruction in GVHD, often requires CD4⁺-T cell help [42]; consequently, blockage of MHC II with an inhibitory peptide reduces miHC-disparate GVHD in mice [43]. Alternative methods of MHCII inhibition for the reduction of GVHD have been explored. Previously, we have shown that chloroquine treatment of mice inhibits T cell responses to MiHC and reduces the development of a miHC-disparate model of GVHD [1]. Interpretation of this data is complicated by the fact that chloroquine can also suppress proinflammatory cytokine secretion from APCs in response to stress or innate immune challenge [44-46] and can block sensing of CpG-DNA [47,48]. We thus sought to verify the contribution of MHC II-mediated antigen presentation to the development of miHC-disparate GVHD by directly inhibiting cathepsin S.

Recent studies have shown that inhibition of cathepsin S in vivo alters antigen presentation and immunity and may be useful as a potential therapy in autoimmune diseases [8,17,18]. However, the importance of cathepsin S appears to depend on mouse strains. The role of cathepsin S in the recognition of endogenous antigens, especially allo-antigens, is still controversial [8,9]. Although cathepsin S treatment impaired the ability of C57BL/6 mice to prime T cell responses against miHA-incompatible BALB.B splenocytes (Figure 4), it did not reduce the severity of GVHD in the same model (Figure 5) or in even a model that relies solely on CD4⁺-T cells to induce disease (Figure 6). Korngold et al [49] reported that severe acute CD4⁺ T cell-mediated GVHD across miHA barrier depends on the expression of nonhematopoietically, rather than hematopoietically, derived alloantigens for target-tissue infiltration and injury in this mice model. This may explain the discrepancy between the effect of LHVS in MLR and GVHD.

Experiments in CatS-deficient mice show that the dependency of antigen presentation on CatS-mediated Ii-chain degradation can vary with both antigen and type of APC [8-10]. In the C57BL/6 → BALB.B aGVHD, despite estimated differences of more than 29 miHA loci between these two strains, the CTL response is thought to be limited to only a few immunodominant Ags [50]. Inhibition of CatS may not affect the particular antigens driving aGVHD in this model. In addition, inflammatory cytokines

produced as a consequence of allogeneic transplantation may be responsible for much of the pathology in this acute model. The failure of CatS inhibition to reduce the severity of aGVHD despite successfully decreasing alloantigen responses may indicate that our previous reduction of mouse GVHD development with chloroquine in the same model [1] may be because of chloroquine-mediated inhibition of TLR9 signaling and the ensuing lack of B cell activation. Previous results in our lab show that GVHD is associated with a population of B cells that express high levels of TLR9 and respond to CpG with increased CD86 levels, suggesting that these B cells may be important APCs in GVHD development [5].

IFN- γ produced by donor T cells is thought to be a central mediator of GVHD (reviewed in [51]); recently, we have shown that early onset cGDHV is associated with decreased levels of INF- γ in humans [52]. However INF- γ and TNF- α are both unlikely to be significant mediators of exacerbated GVHD after cathepsin inhibition, as no difference in the serum levels of these cytokines was observed in the LHVS-treated versus untreated group. Also, development of effective Tregs from the naive T cells used to induce disease could be impeded by LHVS and increase pathology. Although no differences were observed in the relative amounts of Tregs between the two groups, a definite conclusion regarding Treg involvement would require functional assays. A recent article indicates that blocking Cathepsin S activity may increase the amount of endosomal peptides cross-presented on MHCI [53], which could interact with newly formed CD8⁺ T cells. Such an effect may speed up the insidious onset of the cGVHD-like model. Further experiments are needed to clarify the mechanisms behind these observations, and as we cannot rule out off-target effects of LHVS, these observations should be confirmed with alternative Cathepsin S inhibitors. A model that has a less rapid deterioration of the LHVS-treated mice and that allows distinction of donor and recipient cells would be more suited for mechanistic studies. Pharmacologically viable cathepsin S inhibitors are sought after for their potential of therapeutics in a wide range of diseases. Of the many developed, only a few have shown promise in preclinical or clinical trials [54]. However, some have emerged in the past few years and are being clinically evaluated for treatment of arthritis and pancreatic cancer (reviewed in [54]); prophylaxis or treatment of GVHD with these inhibitors should be approached with caution.

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